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The Sulfhydryls of Firefly Luciferase Are Not Essential for Activity†

S. C. Alter‡ and M. DeLuca*
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
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ABSTRACT: Firefly luciferase, containing an average of seven free sulfhydryls per two 50 000-dalton polypeptides, was modified by various sulfhydryl reagents. The differential reactivities of the sulfhydryls in luciferase protected by substrates allow one to define three categories of these groups: Class SH-III contains three sulfhydryls that are not involved in enzymatic activity. Class SH-II contains two sulfhydryls whose modification by different reagents causes varying effects on activity ranging from 0 to 60% inactivation. These sulfhydryls are not essential but may be important structurally or sterically. Class SH-I contains two sulfhydryls that are protected by substrates, either dehydroluciferyl adenylate or dehydroluciferin alone, and are located at or near the active site. The SH-I sulfhydryls are vicinal in the enzyme as demonstrated by their ability to form a disulfide bond. They have also been shown to exist on a single polypeptide chain. Modification of the SH-I groups by most reagents results in complete loss of enzymatic activity; reaction with methyl methanethiosulfonate produces an enzyme that emits only red light whereas native luciferase emits yellow-green light. Evidence is presented that the modified enzyme, while catalytically active, has a distorted active site. It is concluded that these two SH-I sulfhydryls are not essential for activity.

The following reactions are catalyzed by firefly luciferase:[1]

$$E + LH_2 + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\text{–}LH_2AMP + PP_i \quad (1)$$

$$E\text{–}LH_2AMP + O_2 \rightarrow E\text{–}P + AMP + CO_2 + h\nu \quad (2)$$

$$E + L + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\text{–}LAMP + PP_i \quad (3)$$

Reaction 1 is an activation step resulting in the formation of the enzyme-bound adenylate of luciferin. Reaction 2 is the oxidative and light-emitting step. The product oxyluciferin

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‡ Present address: Department of Immunology and Connective Tissue Diseases, Medical College of Virginia, Richmond, VA 23298.

[1] Abbreviations: LH_2, luciferin; L, dehydroluciferin; LAMP, dehydroluciferyl adenylate; pMB, *p*-mercuribenzoate; MMTS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; IAAm, iodoacetamide; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate; SDS, sodium dodecyl sulfate; diamide, 1,1′-azobis(*N*,*N*-dimethylformamide); TEMED, tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid.

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remains tightly bound to the enzyme, resulting in product inhibition. Reaction 3 shows that dehydroluciferin (L), an analogue of luciferin, will undergo the activation step but cannot be oxidized. Only one E–LAMP is former per 100 000 daltons (Denburg & McElroy, 1970), but two molecules of ATP are required for the formation of one LAMP (DeLuca & McElroy, 1984). This is significant because luciferase is catalytically active as a 50 000-dalton monomer (Travis & McElroy, 1966). Thus, the formation of one LAMP per 100 000 daltons implies either that the two monomers are nonidentical or that two identical subunits interact such that only one is catalytically active at any one time.

Further evidence for two sites comes from kinetic experiments where it was found that there are two catalytic sites with different K_m's for ATP–Mg^{2+}. These were defined as I_0, the site resonsible for the initial peak light intensity, and I_1, a site that produces a lower level of light and slowly turns over (DeLuca & McElroy, 1984).

DeLuca et al. (1964) found that luciferase contains six to eight sulfhydryls per 100 000 daltons. After complete reaction with *p*-mercuribenzoate, all of the catalytic activity is lost. However, in the presence of excess L and Mg–ATP to form E–LAMP, two sulfhydryls are protected from reaction with pMB, and the catalytic activity is maintained.

Lee and McElroy (1969) extended these studies using other sulfhydryl reagents. They observed that dehydroluciferin alone will protect the enzyme from inactivation by certain reagents.

Experiments are reported here on the modification of luciferase sulfhydryls with several different reagents. One of these, MMTS, produces an enzyme that emits red light rather than the yellow-green emission that is obtained with the native enzyme. It is further shown that these two sulfhydryls at the active site are vicinal and exist on a single polypeptide chain.

Materials and Methods

Chemicals. Luciferin (LH_2) and dehydroluciferin (L) were synthesized as described by Bowie (1978). DTNB was a product of P-L Biochemicals. Diamide, sodium dodecyl sulfate, sodium pyrophosphate, MMTS, pMB, NEM, and iodoacetamide were obtained from Sigma. Dithiothreitol was purchased from Boehringer Mannheim; acrylamide, bis(acrylamide), TEMED, and ammonium persulfate were Bio-rad; Sephadex G-50 fine was from Pharmacia. Spectrapor 4 dialysis tubing was washed in hot 5% sodium bicarbonate and 1 mM EDTA, rinsed extensively, and stored at 4 °C in 0.01 M β-mercaptoethanol and 0.01 M EDTA. Urea was recrystallized twice from hot ethanol.

Enzyme. Three times recrystallized luciferase was prepared according to the procedure of Green and McElroy (1956). The results reported in this paper were obtained from several preparations of purified luciferase containing 6.8–7.3 free sulfhydryls/mol (100 000 daltons) as measured by the DTNB assay. Luciferase activity was measured on a Turner Monolight 401 photometer attached to an Aminco chart recorder. The assay mix contained enzyme, 0.01 M $MgSO_4$, 1×10^{-4} M LH_2, and 0.025 M glycylglycine, pH 7.8 in a volume of 400 μL; the reaction was initiated by injection of 100 μL of 0.02 M ATP (pH 7.8). Unless otherwise stated, peak light intensity (I_0) was used as the measure of enzymatic activity. All assays were carried out in triplicate, and the average value was recorded.

Determination of Protein. Protein concentration was determined by absorbance at 280 nm, where 1 mg/mL luciferase in a 1-cm cell has an absorbance of 0.75 on the basis of the dry weight (Green & McElroy, 1956). In cases where other absorbing material was present with luciferase, a modified Lowry procedure (1951) was employed with dilutions of pure luciferase as standards.

Determination of Free Sulfhydryls. A 10 mM solution of DTNB in 0.1 M phosphate buffer, pH 7.0, was used. Exact concentrations of DTNB stock solutions were determined in two ways: (a) the absorbance of a 1:100 dilution in 0.1 M phosphate, pH 8.0, was measured at 323 nm, E_{323} = 17 500 M^{-1} cm^{-1} (Yun & Suelter, 1978); (b) 10 μL of DTNB stock and 15.4 mg of DTT were mixed in 10.0 mL of 0.1 M phosphate, pH 8.0. The amount of TNB released was determined by absorbance at 412 nm with E_{412} = 13 600 M^{-1} cm^{-1} (Ellman, 1959). The two values differed by less than ±5%, and their average was taken as the actual concentration. Absorbance measurements were performed on either a Cary 219 or a Hitachi 100-10 spectrophotometer at room temperature. Total protein sulfhydryls were determined by measuring the increase in absorbance at 412 nm in 0.1 M phosphate plus 6 M urea, pH 8.0, with a 50-fold excess of DTNB over protein.

Preparation of NEM-Modified Luciferase and IAAm-Modified Luciferase. Active NEM-modified luciferase was prepared as follows: native luciferase (2 mg/mL) in 0.1 M phosphate, pH 7.0, was incubated with 3×10^{-4} M dehydroluciferin, 1.2×10^{-4} M ATP, and 4.9×10^{-3} M $MgSO_4$ for 15 min. NEM was added to a final concentration of 3.3×10^{-2} M, a 2000-fold excess over E, and incubated at 4 °C for 24 h. The reaction was stopped by addition of DTT to a final concentration of 33 mM. The mixture was then passed through a Sephadex G-50 column (16 cm × 2.5 cm) equilibrated with 0.1 M phosphate buffer, pH 8.0, containing 2 mM $MgSO_4$ and 1 mM pyrophosphate, which facilitates release of enzyme-bound LAMP (Rhodes & McElroy, 1958). One-milliliter fractions were collected. $MgPP_i$ was removed from the protein fractions by dialysis overnight against three changes of buffer. IAAm-modified luciferase was prepared in the same way except a 400-fold excess of iodoacetamide was allowed to react with the enzyme at pH 8.5 for 4 h at 25 °C.

Preparation of S-Methylluciferase. A 2 mg/mL luciferase sample in 0.1 M phosphate, pH 7.2, was incubated with 5 mM MMTS at 25 °C for 3 h. The reaction was stopped by passage through a Sephadex G-50 column. *S*-Methylluciferase contains no free sulfhydryls. The free sulfhydryl content of the modified enzyme did not change for several days, indicating that the mixed disulfides formed during the reaction are fairly stable at pH 7–8 and 4 °C. The concentration of MMTS solutions was determined by reaction with TNB^-, as measured by a decrease in absorbance at 412 nm.

Rate of Modification of Luciferase Sulfhydryls. (*A*) *DTNB.* The specified amount of DTNB was added to 1 mg/mL NEM-modified luciferase in a temperature-controlled spectrophotometer cell set at 25 °C. A pH of 7.0 was used to slow the reaction to a measurable rate. Absorbance was monitored continuously, and samples were removed at various times for assay of enzymatic activity. Aliquots to be assayed were diluted 400-fold in cold 0.1 M phosphate, pH 7.0.

(*B*) *Diamide.* NEM-modified luciferase was used for diamide inactivation to minimize the chance of forming nonspecific cross-links between luciferase molecules. The procedure for following diamide inactivation was essentially the same as that for DTNB inactivation except that the pH was kept at 8.0 and the reaction was incubated in a 25 °C water bath. Diamide was added in a 2- or 10-fold molar excess, and the rate of inhibition was monitored for 2–3 h.

(*C*) *p-Mercuribenzoate.* A stock solution of pMB was made in 0.1 M phosphate, pH 7.1. The exact concentration was determined by measuring the absorbance at 232 nm of a 1:10

dilution, E_{232} = 16 900 M^{-1} cm^{-1} (Boyer, 1954). Luciferase, 5.0 nmol, with or without substrates was mixed with a 5 or 10 μL aliquot of the pMB solution. After 30 min the absorbance at 250 nm was read, and a 10-μL sample removed for assay. Aliquots of pMB were added until no further change in absorbance was detected.

(D) N-Ethylmaleimide. Reaction of luciferase with NEM was performed in 0.1 M phosphate, pH 7.2. The concentration of NEM solutions was determined by measuring the absorbance at 302 nm where E_{302} = 602 M^{-1} cm^{-1} (Means & Feeny, 1971). During the modification, 0.20-mL samples of protein were removed at specified times and then passed over a Sephadex G-50 column (17 cm × 1.2 cm), and one 1-mL fractions were assayed for free sulfhydryl content with DTNB.

(E) Methyl Methanethiosulfonate and Iodoacetamide. Modifications by MMTS and IAAm were done by a procedure essentially the same as that described for NEM at pH 7.0 and 8.5, respectively. The concentrations of these reagents were determined by their reaction with TNB^-; the decrease in absorbance at 412 nm was measured.

Polyacrylamide Gel Electrophoresis. Polyacrylamide slab gel electrophoresis in SDS was carried out in a 7.5% gel with the discontinous system described by Laemmli (1970). The protein molecular weight standard MW-SDS-200 kit was obtained from Sigma. This kit contains the following proteins: carbonic anhydrase, 29 000 daltons; egg albumin, 45 000; bovine albumin, 66 000; phosphorylase B, 97 400; β-galactosidase, 116 000; myosin, 205 000. These were denatured by heating at 95 °C for 5 min in a mixture containing 1% β-mercaptoethanol, 1% SDS, and 0.01 M sodium phosphate. Unmodified and diamide-modified NEM-modified luciferase samples were denatured in a mixture of 5 M urea, 0.05 M sodium phosphate, and 1% SDS. Heat and reducing agents were avoided to preserve disulfide bonds. Electrophoresis was performed at a 20-mA current for 2 h; the gel was stained in 0.05% Coomassie Blue and destained as described by Cooper (1977).

Fluorescence Measurements. Fluorescence emission spectra of dehydroluciferin were obtained on a Perkin-Elmer MPF-44A fluorescence spectrophotometer, with excitation at 350 nm.

Bioluminescence Emission Spectra. Emission spectra of the luciferase-catalyzed bioluminescence were measured in the fluorometer with the excitation lamp turned off. Emission from the bioluminescence reaction was recorded from 520 to 620 nm, with a 10-nm slit width. Emission was scanned at 60 nm/min after the emission intensity was determined to be constant.

Results

Classes of Luciferase Sulfhydryls. Luciferase in the presence of excess L and Mg-ATP (E–LAMP) was allowed to react with different sulfhydryl reagents, and the loss of activity and sulfhydryls was measured. Figure 1 shows the data obtained from reaction of E-LAMP with NEM and IAAm. IAAm rapidly reacts with three sulfhydryls with no loss of activity. There is no further reaction over the next 24 h. NEM also modifies three sulfhydryls within 1 h with no loss of activity. During the next 22 h, two more sulfhydryls are slowly modified with a corresponding loss of 60–70% activity.

If E–LAMP is reacted with either pMB or MMTS, similar results are obtained: two sulfhydryls are protected by LAMP, and the modified enzyme retains 30–50% of the initial activity. These results are summarized in Table I. This information allows us to classify the E–LAMP sulfhydryls according to their differing reactivities to various reagents.

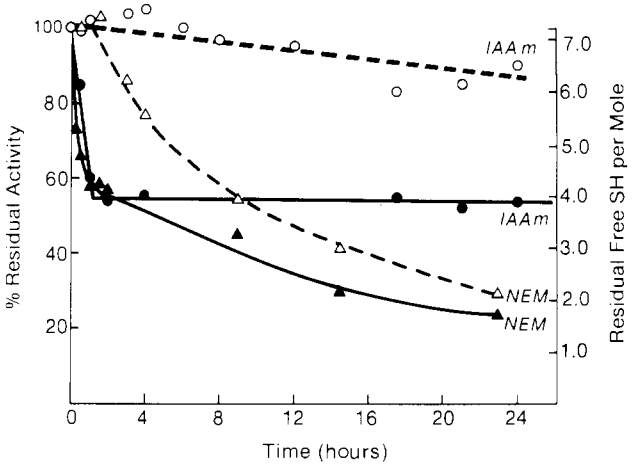


FIGURE 1: Reaction of E–LAMP with NEM and iodoacetamide. The reaction mixture contained in 1.0 mL 1 mg of luciferase, 0.3 mM dehydroluciferin, 1.25 mM ATP, and 5 mM $MgSO_4$ in 0.1 M phosphate buffer; 4.2 mM iodoacetamide (○, ●) at pH 8.5 or 27 mM NEM (△, ▲) pH 7.0 was added and the mixture kept at 4 °C. Enzyme activity (○, △) and sulfhydryl content (●, ▲) were measured over a 24-h period.

Table I: Reaction of E–LAMP Sulfhydryls

reagent	initial free SH/mol	initial activity (%)	SH/mol protected	initial activity protected (%)
pMB	7–8	100	1.98 ± 0.08	26.0 ± 2.0
MMTS	7–8	100	1.92 ± 0.18	46.5 ± 3.5
NEM	7–8	100	2.08 ± 0.02	39.0 ± 2.0
IAAm	7–8	100	3.92 ± 0.05	92.0 ± 7.5

The three sulfhydryls that are modified rapidly by either NEM or IAAm with no loss of activity are class SH-III. Two sulfhydryls are protected to varying extents from reaction with different reagents. Their modification also results in varying degrees of inactivation depending upon the reagent used. These sulfhydryls are designated SH-II. Finally, two sulfhydryls are always protected by LAMP, and their preservation ensures at least partial retention of activity; these are class SH-I. Modification of the SH-I residues by NEM, pMB, DTNB, or IAAm results in complete loss of activity.

The modified luciferases described in Figure 1 will be used to further study the sulfhydryl classes. The IAAm-modified luciferase is like the native enzyme except the SH-III sulfhydryls have been irreversibly modified. The NEM-modified luciferase is about 30–40% as active as native luciferase and contains the two free SH-I sulfhydryls. This enzyme has K_m's for ATP of 1.6 × 10^{-5} and 1.3 × 10^{-4} M, which are the same as for the native enzyme. The K_m for luciferin is 9.6 × 10^{-5} M while, for the native enzyme, this value is 0.4 × 10^{-5} M. Thus, while NEM-modified luciferase is not a perfect model of native luciferase, it is useful for studying the SH-I residues without interference by the other sulfhydryl groups.

Vicinal Sulfhydryls. Two types of experiments support the conclusion that the SH-I sulfhydryl groups are vicinal in the NEM-modified luciferase. Diamide (Kosower et al., 1969), a reagent used to selectively oxidize vicinal sulfhydryls, will completely inactivate NEM-modified luciferase as well as native luciferase. The diamide-inactivated NEM-modified luciferase was subjected to SDS–polyacrylamide gel electrophoresis in the absence of reducing agents to determine if the disulfides formed are intra- or intermolecular. The gel shown in Figure 2 exhibits a single band for the NEM-modified luciferase (lane B). The diamide-inactivated protein has a

Table II: Modification of NEM-Modified Luciferase by DTNB

	initial free SH	TNB^- released/mol	residual activity	residual free SH	TNB^- released/mol by DTT
excess DTNB	2	2.07 ± 0.06	2.6 ± 1.1	0	2.27 ± 0.22
1 equiv of DTNB	2	1.61 ± 0.08[a]	23.1 ± 4.9	0.47 ± 0.10	0.05 ± 0.03

[a] After 3 h of reaction.

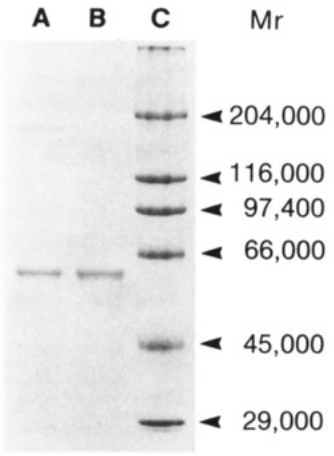
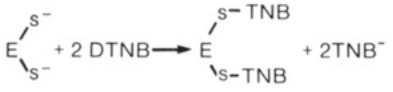
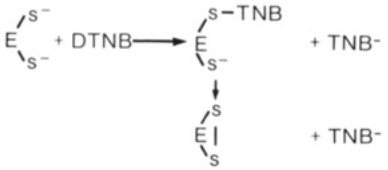


FIGURE 2: SDS–PAGE of diamide-modified NEM-modified luciferase: (A) diamide-modified NEM-modified luciferase; (B) NEM-modified luciferase control; (C) molecular weight standards.

Scheme I



Scheme II



major band migrating in the same position (lane A), and a very faint band is observed corresponding to a small amount of cross-linked protein. Since the diamide inactivation of the enzyme is not due to dimer formation, the two vicinal SH-I groups must be on the same polypeptide.

Table II shows the results obtained when NEM-modified luciferase is reacted with DTNB. If excess DTNB is used, both SH-I groups react with a release of two TNB^-/mol. This enzyme is catalytically inactive. If DTT is added to the column-purified enzyme, 2 equiv of TNB^- is released and enzymatic activity is recovered. These results are consistent with Scheme I. If NEM-modified luciferase is incubated with 1 equiv of DTNB (one DTNB per two free sulfhydryls), 1.6 equiv of TNB^- is released within 3 h, and this enzyme has 20% of the original activity. Thus, the reaction reaches 80% completion in 3 h, and 0.8 equiv of DTNB has reacted producing 1.6 TNB^-. After column purification of the modified enzyme, addition of DTT does not release any bound TNB^-. These results are consistent with the formation of a disulfide as shown in Scheme II.

The initial reaction is the modification of one sulfhydryl to form a protein–TNB mixed disulfide. The vicinal sulfhydryl displaces the TNB forming the disulfide, releasing a second TNB^-. Similar observations have been made by others (Boross, 1969; Flashner, 1972). Figure 3 shows that, if the data are extrapolated to complete inactivation, this would result in the release of 2 equiv of TNB^- from one DTNB.

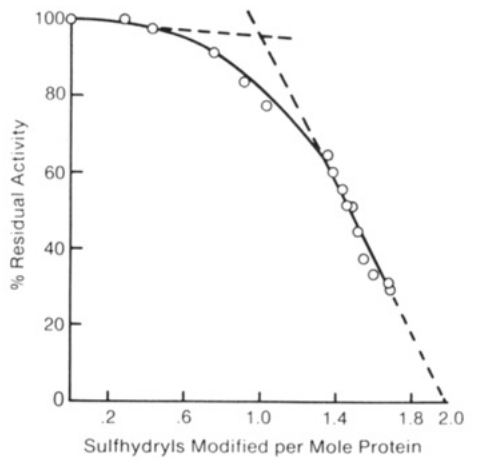


FIGURE 3: Inactivation of NEM-modified luciferase by 1 equiv of DTNB. NEM-modified luciferase, 1.08 mg, was incubated with 1 equiv of DTNB in 0.1 M phosphate buffer, pH 8.0, at 25 °C. Aliquots were removed at specified time intervals, and sulfhydryl content and enzyme activity were measured.

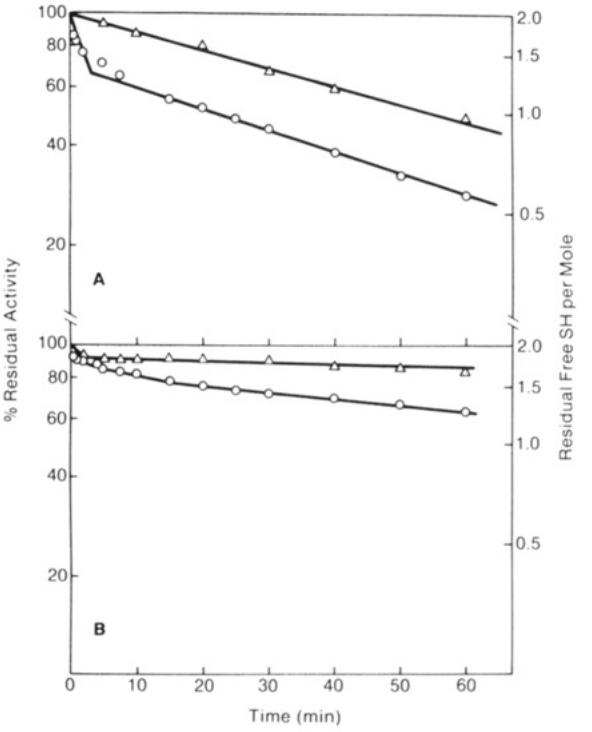


FIGURE 4: Reaction of NEM-modified luciferase in the absence of dehydroluciferin (A) or in the presence of 0.05 mM dehydroluciferin (B). NEM-modified luciferase, 0.45 mg was incubated with 0.05 mM DTNB at pH 7.0. Activity (△) and sulfhydryl content (○) were followed with time.

Protection of SH-I Groups by Dehydroluciferin. Figure 4 shows results obtained when NEM-modified luciferase is reacted with excess DTNB in the absence or presence of dehydroluciferin. In the absence of dehydroluciferin (A), sulfhydryl modification is clearly biphasic. Loss of activity parallels the modification of the second, more slowly reacting sulfhydryl. Figure 2 also gave a similar result. From this, the two SH-I sulfhydryls can be further classified. One is

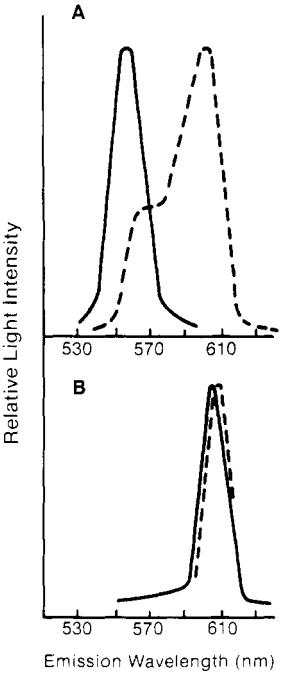


FIGURE 5: Emission spectra of native (A) and *S*-methylluciferase (B). A total of 10 μg of luciferase or *S*-methylluciferase was mixed with excess substrates at pH 8.0 (—) and pH 7.0 (– –). Emission was measured as described under Materials and Methods.

modified first by DTNB with no effect on activity (SH-IB), while the other appears to be more important for enzyme activity (SH-IA). In the presence of dehydroluciferin (B), enzymatic activity is protected, and there is at least partial protection of the sulfhydryls from reaction with DTNB.

IAAm-modified luciferase, which contains both SH-II and SH-I groups, was reacted with excess DTNB with or without dehydroluciferin. Again, only the two SH-I sulfhydryls are protected by dehydroluciferin, indicating the SH-II sulfhydryls are not in the active site.

Modification of Luciferase with MMTS. Reaction of E–LAMP with MMTS results in loss of all but SH-I sulfhydryls and about 50% of the activity. The light emitted by this enzyme is yellow-green. Incubation of native luciferase with an excess of MMTS for 3 h results in an apparent loss of 95% activity and all free sulfhydryls. The light emitted by the modified enzyme is visually red-orange. The emission spectra of native and *S*-methylluciferase at pH 8.0 and pH 7.0 are shown in Figure 5. Native luciferase (A) exhibits a pH-dependent shift from 562 nm at pH 8.0 to a mixture of 562- and 610-nm red emission at pH 7.0 (McElroy & Seliger, 1966). The modified enzyme emits the red light, 605 nm, at both pHs. Reaction of all of the sulfhydryls with pMB, NEM, DTNB, or IAAm resulted in complete inhibition with no evidence of any red light emission.

Corrected Activity of S-Methylluciferase. As mentioned previously, *S*-methylluciferase has an apparent activity of 5% of that of the native enzyme. Two factors contribute to make this an artificially low number. First, the control value (100%) was measured at pH 7.8, where native luciferase emits yellow-green light. The quantum yield for this emission is 0.88 (Seliger & McElroy, 1960). The quantum yield for the red emission is only 0.24 (Seliger & Morton, 1968). Thus, the production of light is only 27% as efficient when red light is emitted, even if the enzyme is working at full catalytic capacity. Second, the Turner 401 photometer used to measure the activity has a Hamamatsu 6199 photomultipler tube, which is about 40% as efficient in the red (605 nm) as in the yellow-green region. Assuming the quantum yield of red light emission for this enzyme is also 0.24, then correcting for these two factors shows the true residual activity of the *S*-methylluciferase is closer to 46%.

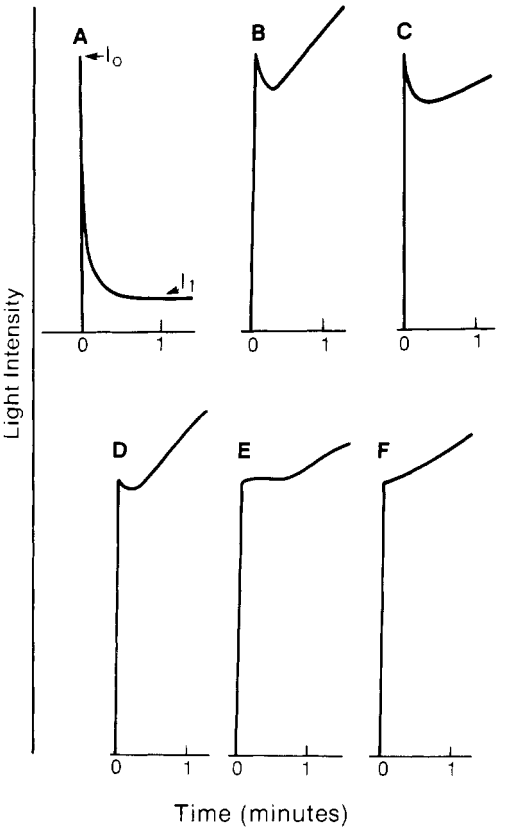


FIGURE 6: Time course of light emission from native luciferase (A) and *S*-methylluciferase (B) in the presence of saturating substrates. The next four curves are obtained with decreasing concentrations of LH_2: (C) 4.3×10^{-6} M; (D) 1.4×10^{-6} M; (E) 9.6×10^{-7} M; (F) 4.8×10^{-7} M. The figures are normalized with respect to the initial flash intensity.

Kinetic Parameters of S-Methylluciferase. Both K_m's for ATP (DeLuca & McElroy, 1984) with the red emitting enzyme are 2-fold higher than those for the native enzyme. The K_m for LH_2 was not determined because of the anomalous kinetics of the modified enzyme. Figure 6A shows a typical chart recording of the time course of light emission from the native luciferase. Injection of ATP produces a flash (I_0) followed by a decay of light to a lower level, I_1. This flash pattern remains the same for all concentrations of LH_2. Figure 6B shows the pattern of light emission observed with excess luciferin and *S*-methylluciferase. This is clearly different from the native enzyme, and at lower concentrations of luciferin (Figure 6C–F), the initial peak of light is essentially lost. Also, there is a gradual increase in light intensity for 10–15 min to a level that is maintained for several minutes before it slowly decays. The K_m for LH_2 for the modified enzyme is not reported, since it is not clear which intensity to measure with decreasing concentrations of luciferin.

Binding of Dehydroluciferin to S-Methylluciferase. Figure 7A shows the fluorescence emission spectrum of dehydroluciferin alone in aqueous solution, pH 7.8. In Figure 7C, native luciferase has been added in a 10-fold excess over dehydroluciferin. In addition to the 550-nm peak, seen with free dehydroluciferin, a new peak at 440 nm is seen. This is due to the phenol form of dehydroluciferin, which exists when it is bound to luciferase (Denburg et al., 1969). Figure 7B shows the fluorescence emission spectrum obtained from a

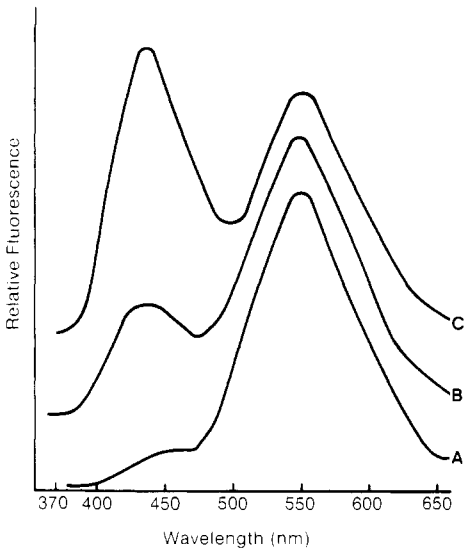


FIGURE 7: Fluorescence emission spectra of dehydroluciferin in the absence or presence of luciferase, performed in 0.1 M phosphate buffer, pH 8.0, in a total volume of 2.0 mL: (A) 14.4 nmol of dehydroluciferin; (B) 2.0 nmol of dehydroluciferin + 20 nmol of *S*-methylluciferase; (C) 2.0 nmol of dehydroluciferin + 20 nmol of native luciferase. All spectra are normalized with respect to the 550-nm emission peak.

10-fold excess of *S*-methylluciferase over dehydroluciferin. The intensity of the 440-nm emission relative to the 550-nm peak is about 60% less than that for native enzyme. A 100-fold excess of *S*-methylluciferase over dehydroluciferin gives an identical emission spectrum, confirming that this represents complete binding of dehydroluciferin to the modified enzyme. These results suggest that modification of the SH-I sulfhydryls, while not sufficient to abolish activity, does cause some distortion of the dehydroluciferin binding site.

Discussion

The results presented here allow us to draw the following conclusions: three classes of sulfhydryls can be defined in E–LAMP. SH-III contains three sulfhydryls that are modified rapidly with IAAm or NEM with no loss of activity. SH-II contains two sulfhydryls that are modified more slowly and are not essential for activity, but since their modification is associated with variable loss of activity, they are probably important either structurally or sterically. SH-I contains two sulfhydryls protected by LAMP or luciferin alone. These are the "active site" sulfhydryls previously described by DeLuca et al. (1964) and Travis and McElroy (1966). These were previously thought to be essential for activity. While modification of these groups with most sulfhydryl reagents does result in complete loss of catlytic activity, it is shown here that reaction of these sulfhydryls with MMTS produces a modified enzyme that emits red light. Clearly, the size of the reagent makes a significant difference at the active site. Similar results were obtained by Smith and Kenyon (1974) with creatine kinase.

Previous studies with various luciferin analogues have lead to the proposed structures for the yellow-green and red emittors, shown in Figure 8 (Hopkins et al., 1967; White et al., 1969). The removal of an additional proton from the monoanion (red emittor) creates the dianion, which emits yellow-green light. It had been suggested previously that there probably was a specific group in the enzyme that accepted the proton and was responsible for the "normal" yellow-green emission (White et al., 1969). These studies strongly implicate one of the SH-I groups as being the proton acceptor.

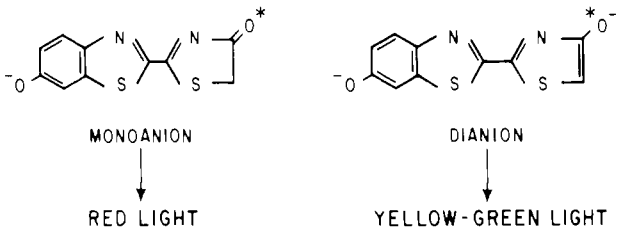


FIGURE 8: Structures of the yellow-green and red emittors.

There are many factors that have been reported to give rise to red light emission: elevated temperatures, decreasing pH, and the presence of certain divalent metal ions (McElroy et al., 1965). In view of the present results, one can suggest that decreasing pH would protonate one or both of the sulfhydryls, which normally aid in the proton removal from the product. Similarly, the various cations are known to complex with sulfhydryls, so these factors can now be explained. The temperature effect is probably a general loosening of the active site not related to any specific residue.

White and Branchini (1975, 1977) used an analogue of luciferin to "ethylate" the enzyme at a nucleophilic residue that was not identified. This ethylated enzyme emitted red light. It is very likely that ethylation occurred at one of the SH-I groups.

In addition to the red light emission, there are several other observations that suggest that the *S*-methylluciferase has a catalytically active but "distorted" active site. There is an apparent increase in the K_m's for ATP, the kinetics of light emission are altered, and the fluorescence emission spectrum for the bound dehydroluciferin is different from the native enzyme.

The increase in K_m's for ATP is small, only about 2-fold, suggesting these binding sites may be only marginally effected. The change in the time course of light emission, Figure 6, can be interpreted to indicate the inhibitory product molecule is dissociating from the altered site more readily than from the native enzyme. Therefore, one does not see the initial flash followed by product inhibition. Similar patterns of light emission have been observed when detergents are added to the reaction mixture, and these were also thought to enhance product dissociation from the enzyme (Kricka & DeLuca, 1982).

The relative decrease in the 440-nm fluorescence emission peak when dehydroluciferin is bound to the modified enzyme can also be explained in a similar way. This 440-nm peak is due to emission from the excited-state phenol form of dehydroluciferin (Denburg et al., 1969). This excited state is severely quenched in aqueous solutions but is protected when the dehydroluciferin is bound in the hydrophobic site of the native enzyme. Therefore, a decrease in this emission when dehydroluciferin is bound to *S*-methylluciferase suggests this site has now become at least partially accessible to water.

When E–LAMP is reacted with MMTS, the two SH-I sulfhydryls are protected and the enzyme retains 40–50% activity. This modified enzyme emits yellow-green light and has normal kinetics. It seems clear that whatever the exact mechanism is which results in red light emission, only the SH-I groups are involved.

Another important aspect of the present studies is the demonstration that the SH-I sulfhydryls are vicinal in the tertiary structure of the NEM-modified luciferase and they are located in a single polypeptide. This must mean that the "active site sulfhydryls" reside on one 50 000-dalton species, but since only one E–LAMP is formed per 100 000 daltons, the other polypeptide must be catalytically inactive. This

heterogeneity has been proposed previously by Denburg and McElroy (1970). All attempts to separate two polypeptides from the apparently homogeneous enzyme have failed. However, knowledge that the two SH-I sulfhydryls do exist in the same polypeptide might serve as a basis for designing future separation schemes.

Registry No. pMB, 2979-65-9; MMTS, 2949-92-0; NEM, 128-53-0; IAAm, 144-48-9; DTNB, 69-78-3; ATP, 56-65-5; LH_2, 2591-17-5; L, 20115-09-7; LAMP, 24404-89-5; luciferase, 61970-00-1.

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Ligand Interactions at the Active Site of Aspartate Transcarbamoylase from *Escherichia coli*†

Paul R. Dennis, M. Vijaya Krishna, Maria Di Gregorio, and William W.-C. Chan*

Department of Biochemistry, McMaster University Medical Centre, Hamilton, Ontario, Canada L8N 3Z5

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ABSTRACT: The active site of aspartate transcarbamoylase from *Escherichia coli* was probed by studying the inhibitory effects of substrate analogues on the catalytic subunit of the enzyme. The inhibitors were chosen to satisfy the structural requirements for binding to either the phosphate or the dicarboxylate region. In addition, they also contained a side chain that would extend into the normal position occupied by the carbamoyl group. All the compounds tested showed competitive inhibition against carbamoyl phosphate. The ionic character of the side chain was found to be highly important in determining the affinity of the inhibitor. On the other hand, very little effect on binding was produced by changing the geometry of the functional group from trigonal to tetrahedral. Our findings suggest that the electrostatic stabilization of the negative charge that develops in the transition state may be a major factor in promoting catalysis. From the available X-ray diffraction data, we propose His-134 as the residue most likely to participate in this interaction. These results have significant implications on the design of reversible and irreversible inhibitors to this enzyme.

Aspartate transcarbamoylase (ATCase)[1] catalyzes the condensation of L-aspartate and carbamoyl phosphate to form carbamoyl-L-aspartate, which is the first intermediate in pyrimidine biosynthesis (Reichard & Hanshoff, 1956). The reaction is crucial for cell division since it provides the required precursors for the production of DNA. Accordingly, the mammalian enzyme is highly active in rapidly proliferating

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[1] Abbreviations: ATCase, aspartate transcarbamoylase (EC 2.1.3.2; aspartate carbamoyltransferase); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IR, infrared; NADH, reduced nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; PALA, *N*-(phosphonoacetyl)-L-aspartate.

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